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FLUORESCENT PROBES IN THE DETECTION OF INSECTICIDES IN WATER

Chester M. Himel

Georgia University

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Fluorescent Probes in the Detection of Insecticides in Water

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FLUORESCENT PROBES IN THE DETECTION OF INSECTICIDES IN WATER

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ABSTRACT

Fluorescent probes are molecules whose spectral responses are changed by their environment. A fluorescent probe which is in equilibrium with the active site of cholinestrace enzymes will have emission spectra and quantum yields corresponding to (a) the free probe and (b) the probe-enzyme complex. Insecticides in water which compete for the active site will displace the probe from its complex and quench the fluorescence of the probe-enzyme complex. The quenching effect will be related to the concentration of the insecticide and the equilibrium constant and quantum yield of the probe-enzyme complex. The change in spectral response can be the basis of a new analytical methodology for insecticides. The objectives of the present research included synthesis of candidate fluorescent probe molecules for cholinesterase enzymes and evaluation of the feasibility of development of a new analytical method for insecticides in water.

Active-site-directed, equilibrium fluorescent probes have been synthesized and used in the development of the analytical system. Results with Dursban, Thiodan, and certain other insecticides are in the range of 1×10^{-7} M. Insecticides which do not compete with, or displace the probe from its complex are not detected. Experimental parameters for design and synthesis of optimum probe molecules were developed.

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SECTION I

CONCLUSIONS

The application of fluorescent probe-enzyme systems to detection of water pollution by insecticides represents a new concept. It can be the basis for the development of a new analytical methodology. The ultimate potentials of the system were not approached because an optimum fluorescent probe molecule was not synthesized during this research. Such an optimum probe appears to be a realistic potential from any extension of the present research. Under presently available conditions, a series of phosphate insecticides can be detected in the range of 10^{-7}M. Sensitivity of the method with an optimum probe molecule appears to be in the range of 10^{-9} molar. The new methodology has proven scientific and analytical feasibility.

SECTION II

RECOMMENDATIONS

It is recommended that further exploratory research be conducted with the aim of design and synthesis of an optimum fluorescent probe molecule for the esteratic site of cholinesterase enzymes. When such a probe molecule becomes available, further research should be done to determine the ultimate sensitivity and scope of the analytical methodology.

SECTION III

INTRODUCTION

Cholinesterase enzymes are irreversibly inhibited by organophosphate and carbamate insecticides. For this reason, many analyses for insecticides use these enzymes as a part of the analytical method. There are two major cholinesterases, acetylcholinesterase (AChE) and serum cholinesterase (ChE). These two enzymes are closely related in that both have an anionic site plus an esteratic site; however, the enzymes are not identical. They differ in their substrate preferences, and in other characteristics. Both of these enzymes were used in this research; however, ChE is the more stable enzyme and, in addition, was available to us in a highly purified, stable state. This made it possible for us to determine whether or not artifacts would be introduced in any use of the commercial (impure) enzyme. Although AChE has been purified, the process is difficult and the pure enzyme is unstable. When the commercial enzymes are used, it is possible that in any given analytical method, one of these enzymes may have specific advantages. Both were studied in much of the research, but ChE was the enzyme of choice.

The inhibition of cholinesterases by organophosphate and carbamate insecticides involves two steps. The first step is the formation of an equilibrium complex, (the Michaelis complex). The second step involves covalent bond formation (phosphorylation or carbamylation) at the esteratic site. These reactions are outlined in equations (1) and (2). The enzyme is represented by E-OH. The insecticide is represented by I-X, in which X represents the alcoholic or phenolic leaving group.

Step 1 $\quad E\text{-}OH + I\text{-}X \underset{}{\overset{K_{eq}}{\rightleftharpoons}} (E\text{-}OH....IX)$ (1)

Michaelis Complex

Step 2 $\quad (E\text{-}OH....I\text{-}X) \xrightarrow{k_2} E\text{-}O\text{-}I + HX$ (2)

inhibited enzyme

K_{eq} = equilibrium constant

k_2 = reaction rate constant

This sequence of reactions is important because it is believed that the mechanism and mode of action of phosphate and carbamate insecticides involves inhibition of cholinesterase enzymes, in vivo. They are also important because any candidate analytical method which involves the enzyme must be related to either the equilibrium in Step 1 or a sequence of reactions involving Step 2.

Inhibition of the enzyme by phosphate or carbamate insecticides has an overall reaction rate which is dependent on both the equilibrium constant (K_{eq}) in the first step and the reaction rate constant (k_2) in the second step. Various organophosphate and carbamate insecticides often differ markedly in the relative values of these two constants; however, it is the esteratic site which is the ultimate point of attack by these insecticides. For this reason, the design for a fluorescent probe preferably includes complex formation at the esteratic site. Complex formation at the anionic site will allow detection of those insecticides which complete for that site.

Many analytical methods for insecticides have been based on the use of fluorogenic substrates. Such fluorogenic substrates are designed to react with various hydrolytic enzymes to produce products which are strongly fluorescent, in a spectral range which is easily measured. This allows measurement of the enzyme-fluorogenic substrate reaction

rate in the presence and absence of insecticides. Unfortunately, measurement of rate constants is subject to ill-defined catalytic and inhibition effects from known or unknown impurities. For this reason, the use of fluorogenic substrates has limitations as an analytical method for insecticides, particularly in water pollution research. For these reasons, it was considered important to develop a new methodology which could take advantage of the sensitivity of fluorescence measurements but which would not be dependent on rate constant measurements. That new methodology is the basis of the present research. It is discussed herein.

Active-Site-Directed, Equilibrium Fluorescent Probes

In 1967, this laboratory started a research program whose objectives included the design and synthesis of new fluorescent molecules as tracer compounds. They were designed with two potential objectives, (1) as a part of the development of new analytical methods for insecticides and (2) for research involving the mechanism and mode of action of insecticides. The analytical methods research was specifically limited to equilibria in enzyme systems to avoid the difficulties inherent in measurement of comparative rate constants.

The objectives of the present research, included the synthesis of active-site-directed, fluorescent equilibrium inhibitors of cholinesterases. These fluorescent molecules could compete with insecticides for the active site of the enzyme. When such fluorescent molecules act as fluorescent probes, changes in spectral responses occur when the probe molecule is removed from the enzyme complex. Therefore, in this new method, the change in spectral response of the probe-enzyme complex could be related to the amount of insecticide present. The sensitivity of the method is dependent on the equilibrium constant of the probe-enzyme complex, the quantum yield of the complex and the degree of interference (if any) of the fluorescence of the free probe. The sequence of reactions is as follows:

When an insecticide in solution competes for the active site of a suitable cholinesterase enzyme-fluorescent probe complex, it will displace the probe from the probe-enzyme complex. This displacement is reflected by a concomitant change in the fluorescence. The analytical methodology therefore involves measurement of change in fluorescence which accompanies insecticide competition for the active site of the enzyme. A suitable fluorescent probe is required. The research under this grant was, therefore, involved in the design, synthesis, and testing of candidate fluorescent probe molecules, study of their spectral responses with cholinesterase enzymes, and determination of feasibility of the analytical method. Some of the research has been published (2-8).

The equilibria involved are illustrated in equations (3) and (4), where P designates an active-site-directed, equilibrium fluorescent probe.

$$E\text{-}OH + P \rightleftharpoons (E\text{-}OH....P) \quad \text{probe-enzyme complex} \qquad (3)$$

$$+ \; I\text{-}X$$

$$P + (E\text{-}OH.....I\text{-}X) \quad \text{insecticide-enzyme complex} \qquad (4)$$

The synthesis of fluorescent substrate analogs is discussed by Himel and Chan (1). A fluorescent substrate analog is a designed, fluorescent molecule which can act as an alternative substrate for the enzyme being used. Their synthesis and application is a bioanalytical problem which involves stereochemical, chemical, structural, solubility, binding and spectroscopic factors.

Fluorescent probes can be excited directly by irradiation, or indirectly by dipole-dipole energy transfer. This latter is a form of sensitized fluorescence (1-2). In either case, the first step in an analytical determination of insecticides involves formation of the probe-enzyme equilibrium complex. The dissociation constant of that complex and the

presence or absence of dipole-dipole energy transfer constitute design parameters which must be studied.

The interaction of chromophores in a fluorescent molecule or its complex can be predicted in general but not specifically. Isolation or interaction of chromophores is an important design parameter. It determines the spectral characteristics of the probe molecule.

The concept of active-site-direction was developed in the early 1960's by several different laboratories (9). It made possible the design of candidate fluorescent probes having a significant measure of selectivity. That selectivity stems from interaction of the probe molecule at the active site of the enzyme rather than at some ill-defined exo area. Interaction and formation of an equilibrium complex at the active site is important because the active-site is the major site of competition with organophosphate and carbamate insecticides.

The importance of fluorescence techniques as research and analytical tools lies in their wide scope and sensitivity. That sensitivity can be orders of magnitude greater than that available from absorption spectroscopy. A fluorescent probe is a fluorescent molecule which is sensitive to its environment. In general, a successful fluorescent probe will have a strong pi-pi* transition, an excited state which is more polar than the ground state and no dominant n-pi* transitions. The latter are generally accompanied by a low extinction coefficient of absorption, a low quantum yield and show extensive intersystem crossing with concomitant phosphorescence.

The development of the new analytical system required active site directed, equilibrium fluorescent probes. Their design and synthesis involves one or more of the following biochemical or spectroscopic parameters:

1. Stereochemical fit at the active site of the enzyme.

2. A dissociation constant of the active-site equilibrium complex in the range of 10^{-5} to 10^{-7}M.

3. An excitation minimum near 280 nm.

4. An excitation maximum near 330 nm or at substantially longer wavelengths when dipole-dipole interaction is not desired.

5. A quantum yield near zero for the free probe in water.

6. A quantum yield of the probe-enzyme complex of 0.5 or larger.

7. An emission maximum of the probe-enzyme complex in the range of 500 nm or longer wavelengths.

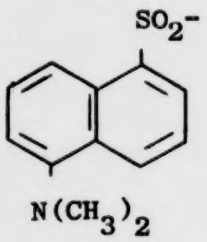
SECTION IV

EXPERIMENTAL

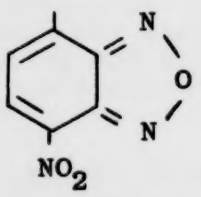
Synthesis of Fluorescent Substrate Analogs as Candidate Fluorescent Probes for Cholinesterase Systems

No known fluorescent probes for cholinesterase enzymes were available when this research was started. The first step in the research program was the synthesis of a wide variety of molecules. Design parameters for suitable fluorescent probes were delineated.

The 5-dimethylaminonaphthalene-1-sulfonyl moiety (1) is readily available and easily introduced into a variety of structures. For this reason it was studied extensively in this research. The NBD moiety (2) is a more recent development in fluorescence research and has valuable spectral characteristics.



(1)



(2)

Candidate fluorescent probe molecules can be designed with bridge systems which isolate the fluorescent moiety or which allow electronic interaction (1-2). The spectral results of isolation of chromophores and electronic interaction have been discussed in detail (1). The structural factors are indicated in (3) and (4). In (3) the groups A and B are separated by the bridge system X and Y, as indicated, such that electronic interaction of A and B takes place. This interaction is reflected in the composite nature of the resulting spectra. In (4) however, A and B moieties are separated by a methylene-type bridge which serves to isolate A and B. In this case, only dipole-dipole (sensitized fluorescence) can occur, and the interaction is limited by distance and mutual orientation (1). In (3) the groups X and/or Y can include (a) aromatic rings, (b) conjugated double bond systems

A-X-Y-B

(3)

$A-CH_2-Y$

(4)

(c) atoms such as oxygen, sulfur, or nitrogen with lone-pair, nonbonding electrons, or (d) -CO-, -SO-, $-SO_2-$. In general, the hydrogen atoms of the CH_2 group in (4) can be replaced by any group which does not circumvent the isolation or one or both can be replaced by alkyl groups.

Synthesis details have been published (2-6). Data are collected in the following Tables. Synthesis data on an extensive series of compounds are described. Many are new compounds and all were synthesized to spectroscopic purity requirements. All compounds were tested for photolytic stability. Critical design parameters exist which determine the utility of candidate fluorescent probe molecules synthesized for use in the analytical system.

Reagents. Dansyl chloride (mp 68-70°C) was obtained from Peninsular Chemresearch. Solvents were reagent grade for synthesis and fluorescent grade for spectral studies. Pyridine was stored over solid KOH prior to use. Tlc film used to monitor synthesis reactions was Eastman, fluorescent and nonfluorescent types.

Analysis. Microanalytical data are by Midwest Micro Laboratories. Structures of new compounds were confirmed by ir

(Beckman IR-10) and nmr (Varian HA-100). In each case the spectra support the structure. We are indebted to Dr. D. R. Leyden for the nmr spectra. All syntheses were monitored by tlc and by ir.

Synthesis of Dansyl Phenolic Sulfonamides. Pyridine Method. Equimolar quantities (usually 0.004 mol) of the aminophenol (or salt) and dansyl chloride (usually 1.1 g) were added to 15 ml of dry pyridine. The reaction was kept under nitrogen and stirred over a 24-hr period. Ether and water were added and the ether layer was separated. The combined ether extracts were washed with 1% HCl and with water to remove traces of pyridine, dried over drierite, filtered, and ether was removed *in vacuo*. The reaction was monitored by tlc (benzene-acetic acid 75/25). Data on sulfonamides are shown in Table II.

Synthesis of Dansyl Sulfonates from Phenols. The phenol (0.02 mol) was added to dansyl chloride (0.02 mol) in 300 ml of ether containing 50 ml of triethylamine (free of dimethylamine). The reaction was monitored with tlc. Reaction times averaged 4 days. The ether solution was washed with water and then dried over drierite. The ether was removed *in vacuo*. The product was recrystallized as indicated. Data are given in Table III.

Synthesis of Fluorescent Carbamates. N-Methylcarbamates were prepared by reaction of methylisocyanate with dansyl phenolic sulfonamide (or oxime). Secondary sulfonamides containing a phenolic OH can react to give the carbamate or the urea carbamate. The former is formed in the absence of catalyst in benzene or toluene solution. The latter is formed when triethylamine is used in catalytic quantities. Reaction times were 24 hr. Data are given in Table IV. Data on model compounds are given in Table V. Enzyme inhibition data are given in Table VI.

Most dansyl derivatives exhibit useful fluorescent properties. Dansyl sulfonamides of reactive amines are readily prepared such that binding properties from differences in the sulfonamido substituent can be studied. Important relationships between binding characteristics and structure have been found in the series of compounds reported herein.

The sulfonamido proton appears to have significant biochemical importance in enzyme and protein binding. The protolysis kinetics of the sulfonamido proton have been studied (7).

In order to prepare fluorescent carbamate analogs, pure sulfonamido phenols were required. The synthesis route was from the bifunctional aminophenol, reactive at both amino and phenolic groups. Prior to the development of tlc methods specificity of reaction at the amino group was generally assumed, or byproduct sulfonate was removed or ignored. Most synthetic procedures in which dansyl chloride was reacted with an aminophenol such as p-aminophenol gave a mixture of phenolic sulfonamide (a) amino-sulfonate (b) and sulfonate-sulfonamide (c). The synthesis routes are simple but preparation of spectroscopic grade product was complex. The pyridine method allowed production of pure (a) directly, a unique and unexpected specificity of reaction.

Dansyl sulfonates from a variety of phenols were found to be reasonably chemically stable in water even at elevated temperatures. All sulfonates tested were, however, photolytically unstable to irradiation at the usual excitation wavelength in the range of 340 nm. Dansyl chloride is unstable in all solvents tested, and its fluorescence is quenched by the Cl to a major extent.

Dansyl fluoride is fluorescent in the same general range as other dansyl derivatives. It is an inhibitor of AChE and ChE. The inhibition of cholinesterase enzymes by sulfonyl fluorides has been studied by Fahrney and Gold (10). Excellent evidence from X-ray diffraction studies indicates probably sulfonate formation at the active-site serine OH. Irradiation of the inhibited enzyme would be expected to give 5-dimethylaminonaphthalene-sulfonic acid (dans acid), λ_{max} 515 nm.

Photolytic stability is a critical design parameter for fluorescent probes. p-Substituted phenolic sulfonamides and their carbamate derivatives were less stable than the meta isomers but had acceptable stability. tert-Sulfonamides para to a phenolic OH were grossly unstable.

N-Aminoalkyl-dansylsulfonamide derivatives have been studied as equilibrium probes for the active site of ChE. Probes specific for the anionic site are listed in Table VII. Their synthesis was as follows:

Reaction of dansyl chloride with a 3M excess of N,N-dimethyl-1,3-diaminopropane in ether gave 5-dimethyl-aminonaphthalene-1-sulfonamido-N,N-dimethyl-n-propyl amine (compound 35, Table VII), referred to as TA in the discussion. The aliphatic quaternary derivative (QA-#36) was prepared by dissolving TA in ether containing a 50 fold excess of methyl iodide. The quaternary derivative precipitated immediately. After four hours, the product was removed by filtration and was recrystallized from water, mp 201-202°C. The structure was verified by infrared and NMR spectra. Purity was monitored by TLC.

Synthesis of N-methylacridinium iodide was a modification of published data and was carried out of room temperature by addition of acridine to a solution of methyl iodide in dimethylformamide.

Fluorescence Measurements

Instrumentation. Emission, excitation, and absorption spectra were obtained with a G. K. Turner "Spectro 210." This instrument presents corrected emission and excitation spectra. Samples for fluorescence measurements were temperature controlled to 25±0.1° . pH measurements were made at room temperature with a Radiometer Model 26 pH meter standardized with NBS standard buffer solutions. Lifetime of fluorescence measurements were made using a TRW nanosecond flash unit and decay computer coupled to a dual gun oscilliscope. Appropriate filters (Corning) were used to isolate excitation and emission wavelengths (10).

Chemicals and Solvents. Pure grade 5-dimethylaminonaphthalene-1-sulfonic acid (DnsOH) was from Pierce Chemical Co. It was homogeneous on Eastman tlc film, using benzene-methanol (1:1), and was comparable to that of a highly purified standard.

Et_4PP, p,p'-dichlorophenyl-2,2,2-trichloroethane, p,p'-dichlorophenyl-2,2-dichloroethylene, heptachlor, dieldrin, and Thiodan were of analytical quality and gifts of the Southeast Environmental Research Laboratory, Athens, Ga. Maretin was a gift of the Chemagro Chemical Co. Solvents and reagents were reagent grade or fluorescent quality. All water was double distilled from glass. Buffers were either Tris-HCl (0.05 M) or citrate-disodium hydrogen phosphate (0.1 M). Any pH below or above the capacity of these buffers was obtained with 0.1 N H_2SO_4 or 0.1 N NaOH.

Lyophilized horse serum cholinesterase (ChE) (type IV), bovine erythrocyte acetylcholinesterase (AChE) (type III), electric eel AChE (type III), were purchased from Sigma. Amounts of cholinesterase are given as milligrams of enzyme preparation per milliliter. The bovine erythrocyte AChE hydrolyzed 2.9 umoles of ACh per min per mg. The electric eel AChE hydrolyzed 540 umoles of ACh per min per mg of preparation. Horse serum ChE hydrolyzed 6.0 umoles of ACh per min per mg of preparation.

Purified ChE was prepared by the Method of Main (10) and was used to determine the effect of enzyme purity on the spectral responses of the probe-enzyme complex in the presence of insecticides.

Methods. Fluorometric titrations were performed manually with Hamilton microsyringes. Total volumes of all samples for fluorometric measurements were 2.0 ml. Irradiation of samples containing protein was limited to 5 min. or less to avoid photooxidation of the tryptophan and histidine residues in the protein. When mixing was necessary, cells with fitted Teflon stoppers were used. Dissociation constants (K_d) were determined graphically after the methods of Chen and Kernohan (11), Chen (12), and Jun et al. (8).

Cholinesterase activities were determined titrametrically through a modified method of Nabb and Whitfield (13) using 0.005 N NaOH as the titrant. The titration was at pH 7.3 ChE solutions were made up in double-distilled water without salt additives.

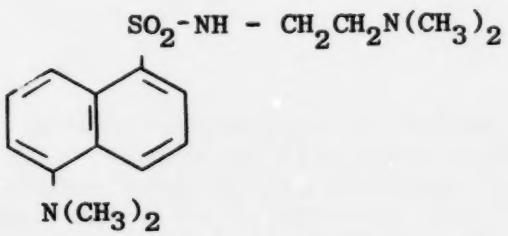
The comparative methods of Parker and Rees (14) were followed in quantum efficiency calculations. The equation used in calculating quantum efficiency has been given by Fletcher (15). Quantum efficiencies of probe-enzyme complexes were determined in aqueous systems. DnsOH was used as the quantum yield reference standard with the quantum yield taken as 0.36 in 0.1 m $NaHCO_3$. All solutions which did not contain protein were purged with special purity nitrogen to retard oxygen quenching. Fluorescence quality sample cells (Hellma) 1.0 cm in path length with Teflon stoppers were used. Where absorption was a problem, 0.3-cm microcells with special cell holders were used (AMI-NCO). Peak areas were integrated with a Hewlett Packard 3370A electronic digital integrator interfaced with the spectrofluorometer.

Enzyme Inhibition Data

In order to be useful, a candidate fluorescent probe molecule must be an equilibrium inhibitor of the enzyme active site. Inhibition data are given in Table VIII, from pH Stat measurements.

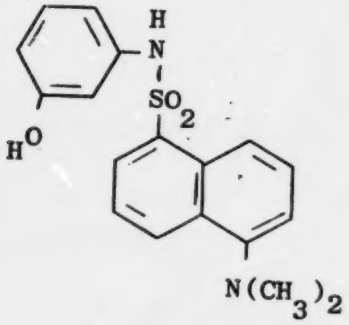
Fluorescence Data with Insecticides

The fluorescent probe molecule TA



was studied in considerable detail. It is a competitive inhibitor of ChE and is relatively specific for ChE. Since it also reacts with phosphorylated enzyme to form a complex, it is assumed that the probe molecule binds (1) at the anionic site and (2) at an exo area adjacent to the anionic site. Because it does not specifically interfere with the esteratic site, phosphate and carbamate insecticides which have a minimum bulk do not interfere with the probe-enzyme complex. On the other hand, with commercial enzyme, most chlorinated hydrocarbon insecticides interfere with the probe-enzyme complex, presumably at some exo area. This extension of the methodology was unexpected and is limited to the commercial enzyme. It is not present when the pure enzyme was used.

Quantitation of the decrease in fluorescence intensity of the TA-ChE complex as a function of various insecticides has been studied. The data are discussed in the following section. Attempts to prepare a suitable fluorescent probe specific for the esteratic site included the phenolic sulfonamides below, its <u>para</u> isomer and their carbamate esters.



In this series of compounds, both the meta and para isomers are readily available by simple synthesis steps using the selective pyridine method developed for this compound (6). The fact that compound 3 is a non-competitive inhibitor of the enzyme indicates that its major binding is adjacent to

the esteratic site, thus a suitable ester derivative of this compound should be studied. The carbamate ester of the *para* isomer was obtained and found to have a limited potential because of its relatively low inhibition of the enzyme (cf. Table VI).

Analytical Data

The objective of this research was to synthesize one or more fluorescent probe molecules which would be useful in this new analytical methodology for insecticides and to determine the range of sensitivity of the analytical method. Those objectives were realized. Suitable fluorescent probe molecules can be synthesized and the analytical method is feasible. Analytical sensitivity with several insecticides is in the range of 10^{-7} M with the potential for considerable increase in sensitivity when an optimum probe molecule is made available. The design parameters for an optimum probe molecule have been delineated by the research.

A series of phosphate insecticides and other compounds of interest were tested to determine whether they interferred with the TA-ChE complex. The data are given in Table IX. The fact that two organophosphates did not compete with the probe-enzyme complex indicated that this probe was not located at the esteratic site of the enzyme. It was later determined that the binding of this probe was at (1) the anionic site and (2) at a hydrophobic area adjacent to the anionic site. Since none of the phosphate insecticides which did give a positive response are known to bind at the anionic site, it was assumed that their potential detection by this analytical system was dependent on competition for the hydrophobic area occupied by the probe molecule. No carbamate type insecticides were found to respond to the probe enzyme complex, however, the rather bulky cationic carbamate molecule esserine did replace the probe, as evidence by the expected change in fluorescence.

When a series of chlorinated hydrocarbon insecticides were tested, it was not expected that they would affect the probe-enzyme complex. When the commercial enzyme was used, a competitive replacement was found. The experimental results could be duplicated and after extensive research, the effect was considered to be real and not an artifact. Further study of the unexpected effect was made with purified enzyme and here, it was found that chlorinated hydrocarbons did not displace the probe from its enzyme complex. The use of purified enzyme did not alter the fundamental nature of the reaction of organophosphate insecticides with the probe-enzyme complex, hence the effect of chlorinated hydrocarbons relates only to commercial enzyme. It was not possible to determine the absolute nature of the interaction of chlorinated hydrocarbons with the probe-enzyme complex formed with commercial enzyme. It was assumed that the enzyme system contained a protein which was complexed near the active site which furnished a hydrophobic area common to the chlorinated hydrocarbons and the naphthalene moiety of the probe molecule.

In Figures 1-3 the change in fluorescence of the probe-enzyme complex in the presence of methyl parathion, Dursban and Thiodan is shown. The latter indicates the unexpected effect of chlorinated hydrocarbons with the complex formed with the commercial ChE enzyme. Figures 4 and 5, show a family of curves and relate the effect of probe concentration and insecticide concentration for Dursban and for Thiodan. The latter indicates again the reproducibility of the competitive effect with chlorinated hydrocarbons when the commercial enzyme is used. Thiodan does not affect the probe-enzyme complex formed from TA and the pure ChE enzyme.

SECTION V

DISCUSSION

Choice of Enzyme

The requirement for analytical specificity means that the enzyme for use in this research must have selective reaction with organophosphate and carbamate insecticides. For this reason, acetylcholinesterase (AChE) and serum cholinesterase (ChE) were the only enzymes studied. ChE was the enzyme of choice because of its ready availability as commercial enzyme (about 1-5% enzyme) and the availability to us of pure, stable enzyme (10). The fact that the inhibition of these two enzymes was not identical with each probe molecule indicates that both enzymes may have significant potential in any final study and method development research. Relative inhibition data are given in Table VIII for various probe molecules.

Synthesis of Candidate Probe Molecules

Synthesis and enzyme reaction data are given in Tables I-VIII. Cholinesterases are unique enzymes in that they have two substantially different active sites. The anionic site is specific for aliphatic and quaternary amines and apparently acts as a positioning site for the quaternary moiety in acetylcholine. The esteratic site on the other hand is a hydrolytic site and is the site which is phosphorylated and carbamylated during insecticide inhibition. Utility as a fluorescent probe for this research was found to be determined by the following criteria:

1. The candidate probe molecule must complex with the enzyme with a K_d (dissociation constant) in the range of 10^{-5} to 10^{-7} M. This is a range related to that of many insecticides and allows maximum change in fluorescence of the probe-enzyme complex when insecticide is added.

2. The candidate probe molecule must show competitive inhibition of the enzyme indicating that it complexes at the (a) anionic site, (b) the esteratic site, or (c) both the esteratic and anionic sites.

3. An optimum probe molecule will complex at both the esteratic and anionic sites or at the esteratic site or at the esteratic site and an adjacent hydrophobic area.

4. An optimum probe molecule will have a quantum yield in water which is close to zero and a quantum yield of the probe-enzyme complex which is in the range of 0.5.

5. The optimum fluorescence range for a probe-enzyme complex is in the range of 500-600 nm.

6. The probe must be soluble in water in the range of 10^{-4} molar.

Near the end of the first year of this research program, the probe molecule 5-dimethylaminonaphthalene-1-sulphonamido-N, N,-dimethyl-n-propyl amine (compound 35, Table VII) was developed and found to be a promising probe molecule. The structural formula is given in the experimental section. At that time, it was noted that this probe molecule (given the trivial designation TA) was specific for cholinesterase, and did not react with AChE. After considerable study it was determined that TA binds at the anionic site of ChE and at an adjacent hydrophobic site. Other anionic sites probes were also developed but the objective of development of a probe suitable for the esteratic site was not obtained. Relative solubility was found to be a critical factor and substantially eliminates compound 20 which was an esteratic site probe. Relative solubility also eliminates compound 9. Water solubility in the range of 10^{-4} molar is a critical parameter.

SECTION VI

ACKNOWLEDGEMENTS

The research was carried out at the University of Georgia, Department of Entomology, with Dr. R. T. Mayer and with Dr. L. M. Chan. Synthesis research was carried out principally by Mrs. Wissam Aboul-Saad and Miss Carol Lord.

Table I. Synthesis of Dansyl Phenolic Sulfonamides

Compd and no.	mp, °C	Yield %	Analysis	Calcd %	Found %
1 p-(5-Dimethylamino-naphthalene-1-sulfonamido) phenol	142-4	62	C H N	63.15 5.26 8.18	63.00 5.28 8.05
2 p-N-Methyl-(5-dimethylamino-naphthalene-1-sulfonamido)-phenol	114-5	45	C H N	64.04 5.62 7.86	63.70 5.86 7.79
3 m-(5-Dimethylamino-naphthalene-1-sulfonamido) phenol	165-6	50	C H N	63.15 5.26 8.18	63.27 5.41 8.16

R = 5-(dimethylaminonaphthalene)-1-sulfonyl (dansyl);
R' = H or CH_3.

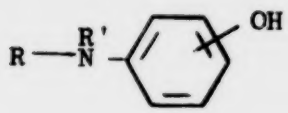


Table II. Synthesis of Dansyl Sulfonamides $RSO_2NR'R''$

Compd and no.	mp, °C	Yield %	Analysis	Calcd %	Found %
4 4-(5-Dimethylamino-naphthalene-1-sulfonamido) methylene-1,2-methylenedioxy benzene	91-3	50	C H N	62.31 5.21 7.28	62.37 5.41 7.18
5 2-(5-Dimethylamino-naphthalene-1-sulfonamido) pyridine	235	50	C H N	62.39 5.20 12.84	62.27 5.31 12.54
6 3-(5-Dimethylamino-naphthalene-1-sulfonamido) pyridine	194	50	C H N	62.39 5.20 12.84	62.39 5.22 12.89
7 N-(5-Dimethylamino-naphthalene-1-sulfonamidoethyl-glycinate	76-7	50	C H N	57.14 5.95 8.33	56.77 5.90 8.23
8 m-(5-Dimethylamino-naphthalene-1-sulfonamido) benzotrifluoride	129-30	70	C H N	57.87 4.31 7.10	57.93 4.43 7.04
9 m-(5-Dimethylamino-naphthalene-1-sulfonamido) ethylbenzoate		68	C H N	63.29 5.53 7.03	63.39 5.56 7.14
10 p-(5-Dimethylamino-naphthalene-1-sulfonamido) benzoic acid	218-20	75	C H N	61.60 4.86 7.56	61.63 4.74 7.43

Table II. (continued)

Compd and no.	mp, °C	Yield %	Analysis	
			Calcd %	Found %
11 m-(5-Dimethylamino-naphthalene-1-sulfonamido) benzoic acid	222-3	80	C 61.62 H 4.86 N 7.57	61.50 4.94 7.57
12 5-Dimethylamino-naphthalene-1-sulfonamide	215	100		
13 N-Methyl-5-dimethylamino-naphthalene-1-sulfonamide	110-1	85	C 59.09 H 6.06 N 10.61	58.81 5.95 10.34
14 N,N-Dimethyl-5-dimethylamino-naphthalene-1-sulfonamide	100-2	85	C 60.43 H 6.47 N 10.07	60.53 6.62 9.86
15 (5-Dimethylamino-naphthalene-1-sulfonamido) benzene	141-2	78		
16 N-Methyl (5-dimethylamino-naphthalene-1-sulfonamido) benzene	94-5	78	C 67.06 H 5.88 N 8.24	66.57 5.94 8.18
17 N-Benzyl-5-dimethylaminonaphthalene-1-sulfonamide	139			

Table II. (continued)

Compd and no.	mp, °C	Yield %	Analysis	
			Calcd %	Found %
18 8-(5-Dimethylamino-naphthalene-1-sulfonamido) quinoline	152-4	58	C 66.84 H 5.04 N 11.14	66.61 5.17 11.22
19 8-(5-Dimethylamino-naphthalene-1-sulfonamido)-6-methoxy quinoline	207-8	73	C 64.86 H 5.16 N 10.32	64.87 5.21 10.39
20 p-(5-Dimethylamino-naphthalene-1-sulfonamido) benzyl phosphonate	162-3	78	C 57.98 H 6.09 N 5.88	57.19 6.10 5.90

Table III. Synthesis of Dansyl Sulfonates from Phenols RSO_2-OR'

		mp, °C	Reaction time, days	Yield %	Analysis Calcd %	Analysis Found %
21	1,2-Dioxymethylene-4-(5-dimethylaminonaphthalene-1-sulfonato) benzene	86-7	1	94	C 61.47 H 4.58 N 3.77	61.33 4.84 3.96
22	5-Dimethylaminonaphthalene-1-(4-nitrophenyl)-sulfonate	114	1	63	C 58.07 H 4.30 N 7.53	58.37 4.41 7.47
23	5-Dimethylaminonaphthalene-1-sulfonato)-3-dimethylamino benzene	131-2	4	54	C 64.86 H 5.95 N 7.57	64.60 5.98 7.40
24	4-(5-Dimethylaminonaphthalene-1-sulfonato) acetophenone	90-2	2	64	C 65.01 H 5.15 N 3.79	64.82 5.20 3.79
25	4-(5-Dimethylaminonaphthalene-1-sulfonato) acetophenone oxime	144-6	1	40	C 62.48 H 5.21 N 7.29	62.48 5.32 7.24

R = 5-(dimethylaminonaphthalene)-1-sulfonyl (dansyl);
R' = substituted phenols

Table IV. Fluorescent Carbamates and Dns-F

Compd and no.	mp, °C	Yield %	Analysis	
			Calcd %	Found %
26 p-(5-Dimethyl-aminonaphthalene-1-sulfonamido) phenyl-N-methyl-carbamate	160-2	67	C 60.15 H 5.26 N 10.52	60.49 5.55 10.28
27 p-N-(N'-Methyl-carbamoyl)-N-(5-dimethyl-aminonaphthalene-1-sulfonyl) phenyl-N''-methyl carbamate	159-61	97	C 57.89 H 5.26 N 12.28	57.82 5.47 12.00
28 p-(5-Dimethylamino-naphthalene-1-sulfonato) aceto-phenone (N-methyl-carbamoyl) oxime	114-5	77	C 59.86 H 5.21 N 9.52	59.58 5.37 9.47
29 p-(5-Dimethylamino-naphthalene-1-N-methylsulfonamido) phenyl-N-methyl-carbamate	50 dec	80		
30 5-Dimethylamino-naphthalene-1-sulfonyl fluoride	50-2	95	C 56.92 H 4.74 N 5.53	57.49 4.90 5.53

Table V. Model Compounds

Compd and no.	mp, °C	Yield %	Analysis	Calcd %	Found %
31 p-Benzensulfonamido-phenol	157-8	88	cf. Tingle and Williams (1907)		
32 p-Benzenesulfonamido-phenyl-N-methyl-carbamate	137-8	100	C H N	54.90 4.58 9.15	54.87 4.58 8.93
33 p-N-Methylbenzene sulfonamidophenol	135-6	81	C H N	59.32 4.94 5.32	59.48 4.98 5.23
34 p-N-Methylbenzene sulfonamidophenyl-N-methylcarbamate	115-7	100	C H N	56.25 5.00 8.75	56.31 5.09 8.81

Table VI. Enzyme Inhibition Studies on Bovine Erythrocyte Acetylcholinesterase (AChE) and Horse Serum Cholinesterase (ChE)

Compd. and no.	Inhibition % of controls[a,b]					
	1×10^{-5} M		5×10^{-5} M		10×10^{-5} M	
	AChE	ChE	AChE	ChE	AChE	ChE
26 p-(5-Dimethylamino-naphthalene-1-sulfonamido)-phenyl-N-methylcarbamate	3±2	6±2	10±2	30±2	14±2	40±4
27 p-N-(N'-methylcarbamoyl)-N-(5-dimethylaminonaphthalene-1-sulfonyl) phenyl-N"-methylcarbamate	0.00	8±5	6±2	13±3	11±1	18±1
28 p-(5-Dimethylaminonaphthalene-1-sulfonato) acetophenone (N-methylcarbamoyl) oxime	10±1	33±2	13±1	43±2	16±1	47±2
30 5-Dimethylaminonaphthalene-1-sulfonyl fluoride	9±3	23±1	18±2	33±3	24±2	35±2
Matacil (4-dimethylamino-m-tolyl methylcarbamate)[c]	79±2	30±4		58±3		

[a]Average of three replicates. [b]Average activity of control for AChE = 10.71 uM of acetylcholine hydrolyzed per hour, and for ChE = 9.13 of acetylcholine hydrolyzed per hour.
[c]Chemagro Crop., Kansas City, Mo.

Table VII.

Aminoalkyl-Dns-Sulfonamides
(Anionic Site Fluorescent Probes)

Compd and no.	mp, °C		
35 5-Dimethylamino-naphthalene-1-sulphon-amido-N,N,-dimethyl-n-propylamine	119-20	C 60.80 H 7.46 N 12.50	60.43 7.23 12.27
36 quaternary iodide of 35	201-2	C 45.28 H 5.87 N 8.80	45.16 5.86 8.73
37 N-Methylacridinium iodide			

Table VIII.

Enzyme Inhibition by Fluorescent Probes

Compound	Conc. (M/l)	Inhibition AChE	Inhibition ChE
9, m-(5-Dimethylamino-naphthalene-1-sulfon-amido) ethylbenzoate	2×10^{-5}	44	
	1×10^{-5}		6
20 p-(5-Dimethylamino-naphthalene-1-sulfon-amido) benzyl diethyl phosphonate	1×10^{-5}	46	21
3, m-(5-Dimethylamino-naphthalene-1-sulfon-amido) phenol	1×10^{-4}	48	41
	2×10^{-4}	64	55
6, 3-(5-Dimethylamino-naphthalene-1-sulfon-amido) pyridine	1×10^{-4}	28	2
quaternary of #6	1×10^{-4}	34	2
8, m-(5-Dimethylamino-naphthalene-1-sulfon-amido) benzotrifluoride	2×10^{-5}	26	14
Dns-N-CH_2COOH H	1×10^{-5}	45	0
11, m-(5-Dimethylamino-naphthalene-1-sulfon-amido) benzoic acid	1×10^{-4}	32	0
Dns-N-$(CH_2)_2N$-$(CH_3)_3$· I H	1×10^{-4}	3	
	2×10^{-5}		37
Dns-N-$(CH_2)_3N(CH_3)_3$· I H (#36)	1×10^{-4}	4	37
Dns-N-$(CH_2)_3$-$N(CH_3)_2$ (#35)	1×10^{-4}	14	
	2×10^{-5}		47

Compound		Inhibition	
		AChE	ChE
37, N-Methylacridinum iodide	5×10^{-7}		58
	1×10^{-6}	58	
	2×10^{-6}	75	85
	2×10^{-5}	100	
5, 2-(5-Dimethylamino-naphthalene-1-sulfon-amido) pyridine	1×10^{-5}		11
7, N-(5-Dimethylamino-naphthalene-1-sulfon-amidoethylglycinate	1×10^{-5}		4

Table IX.

Compounds Which Compete for the Probe-Enzyme Complex Using Probe TA

Insecticide or Compound	Response
Naled	+
Diazinon	+
Pho rate	+
Paroxon	+
Methylparathion	+
TEPP	+
Guthion	+
Ethion	+
Dursban	+
Malathion	+
DDT	+*
Heptachlor	+*
Diedrin	+*
Thiodan	+*
DDE	+*
Esserine	+
Zectran	-
Vapona	-

*commercial enzyme

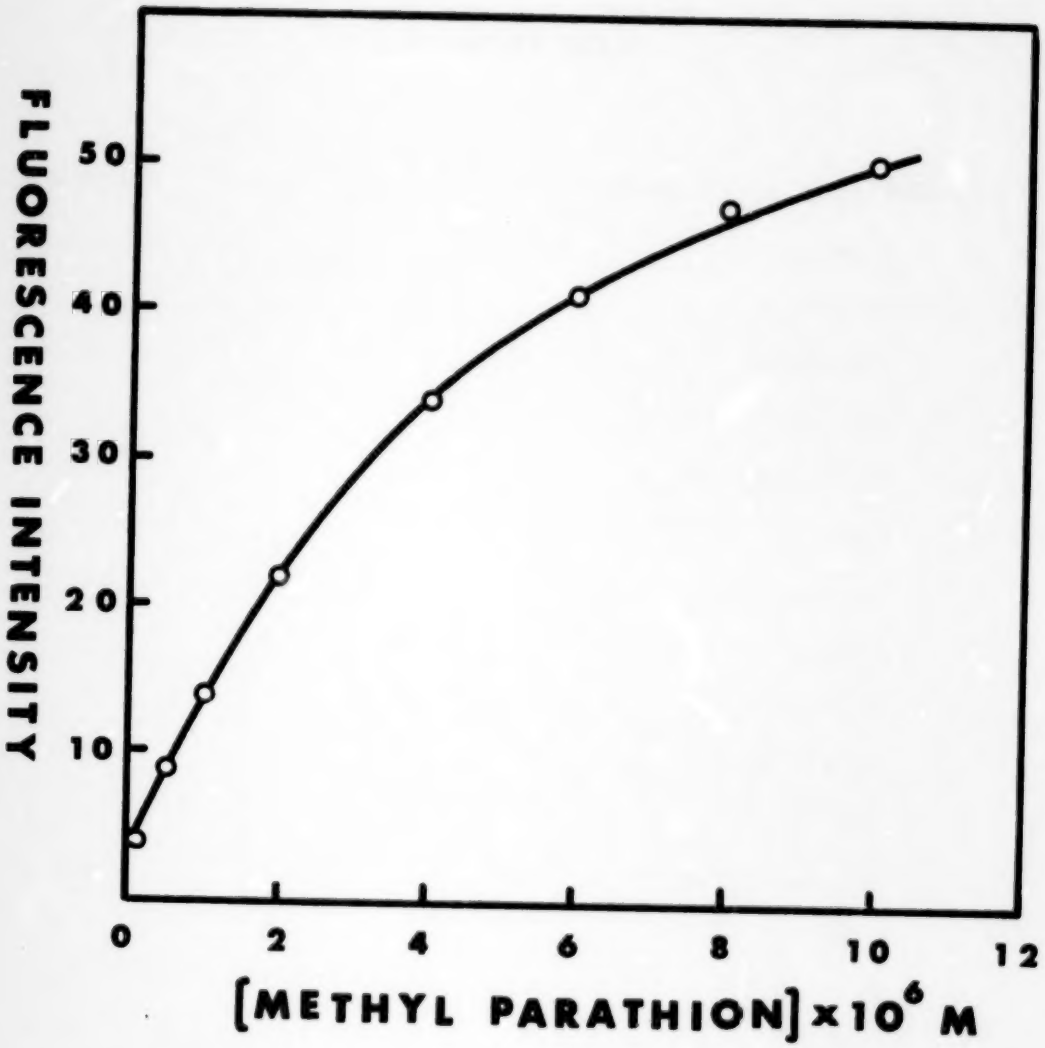


Figure 1

Change in fluorescence of the TA-ChE complex with Methyl Parathion

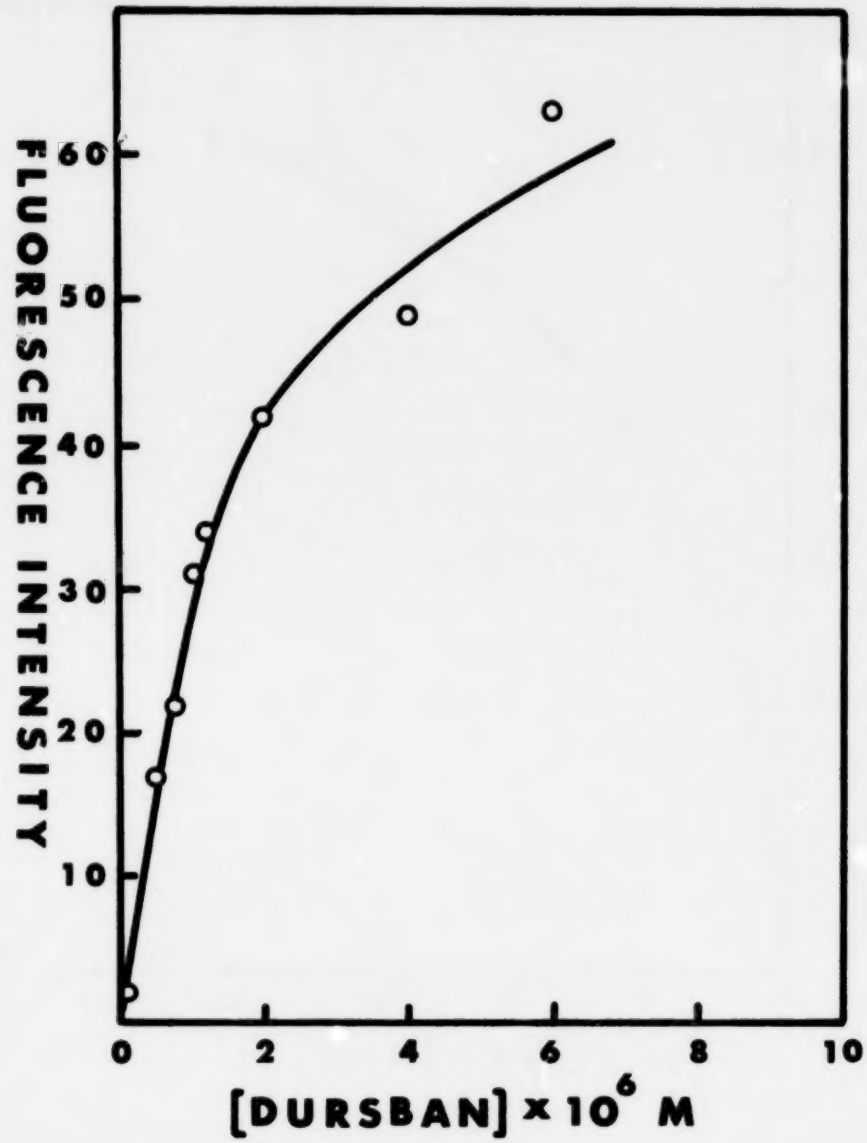


Figure 2

Change in fluorescence of the TA-ChE complex with Dursban

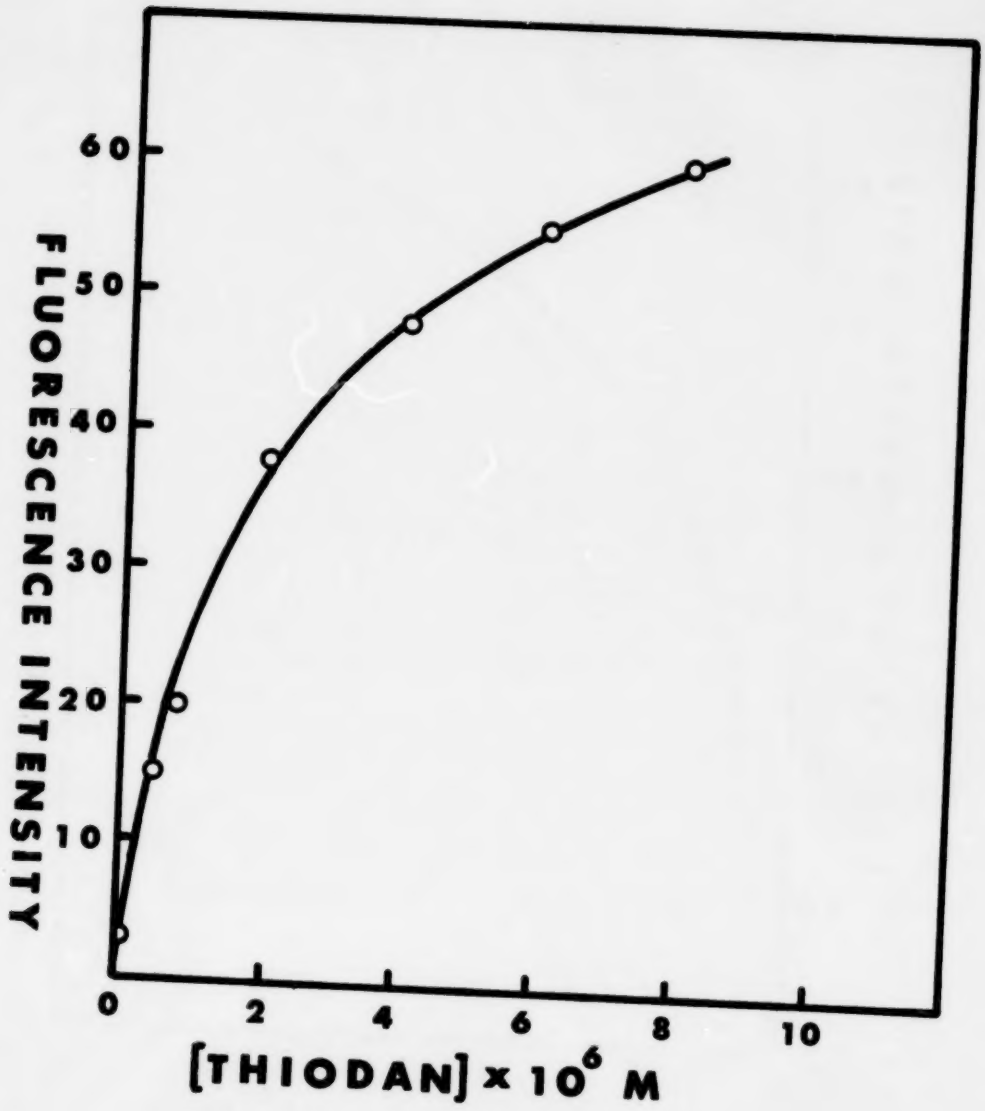


Figure 3

Change in fluorescence of the TA-ChE complex with Thiodan

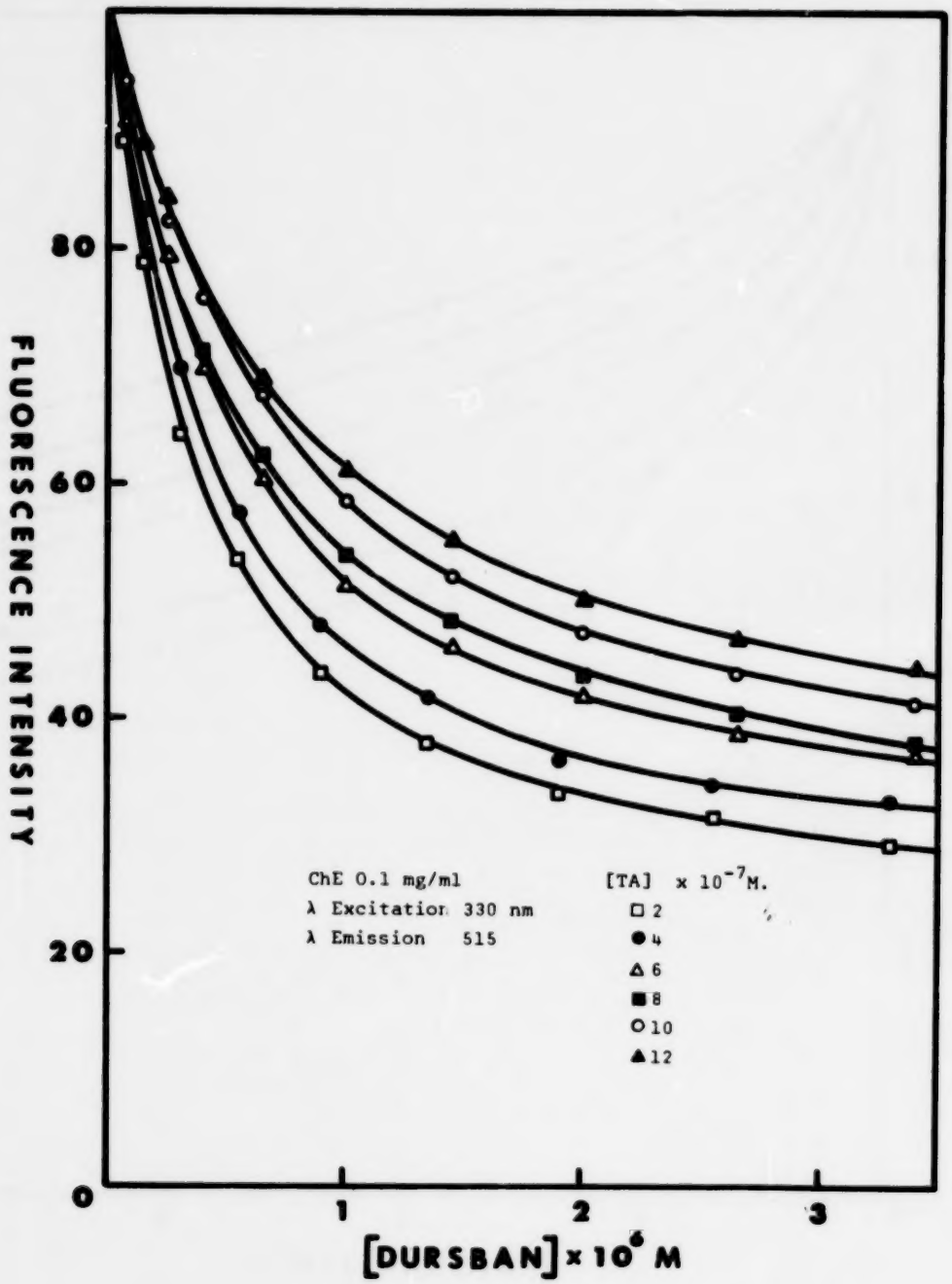


Figure 4

Relative fluorescence effects with Dursban

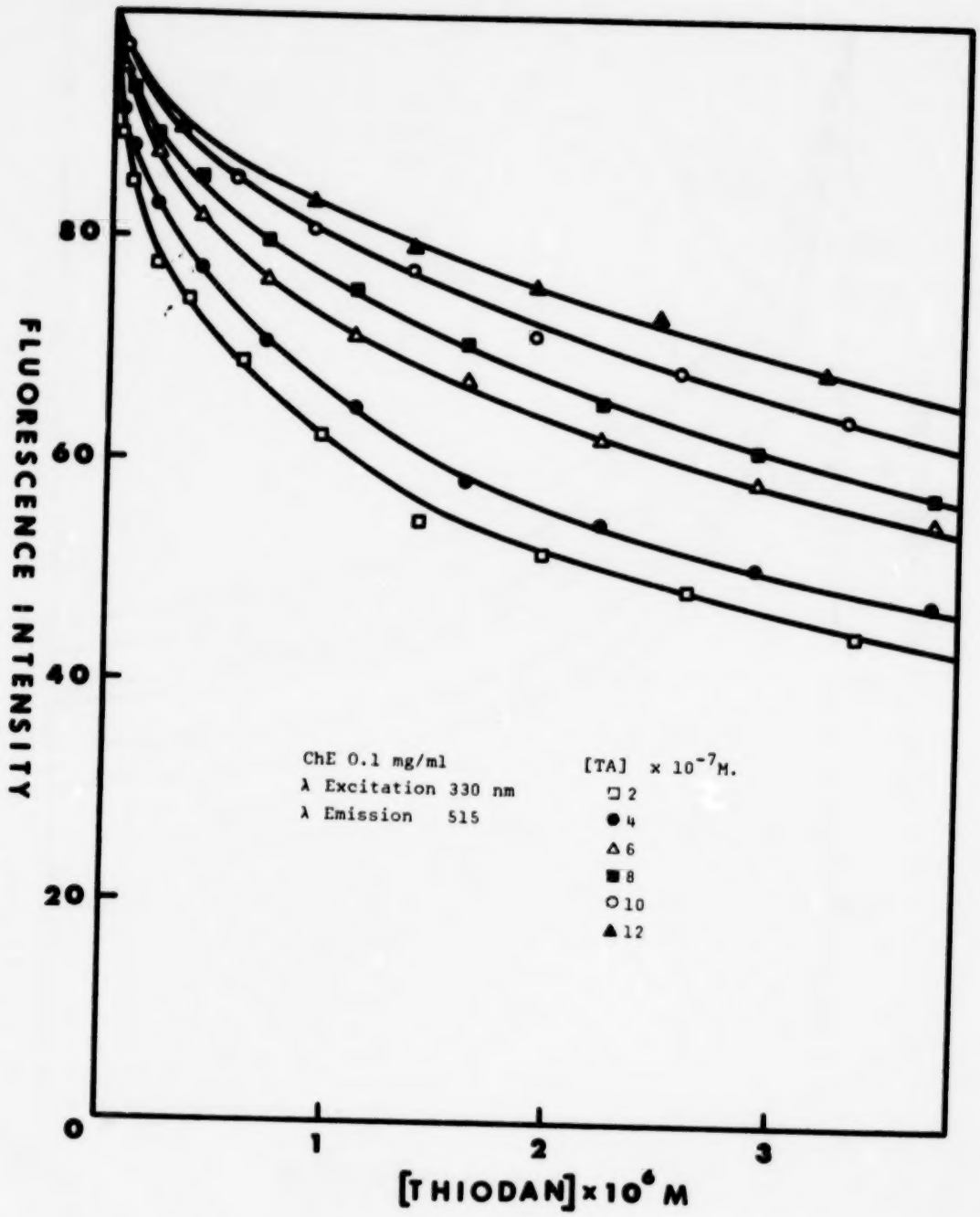


Figure 5

Relative fluorescence effects with Thiodan

SECTION VII

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